



## Effect of optimum processing conditions on total polyphenols, antioxidant activity and FTIR spectra of kodo millet (*Paspalum Scrobiculatum*)

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### ABSTRACT

The various processing treatments like soaking, germination, boiling, steaming, roasting and fermentation are known to affect the chemical composition and improve the nutritive value of foods. Hence the present study was planned with the objectives to optimize the processing conditions and to study the effect of optimized conditions on total polyphenol content, antioxidant activity and FTIR spectra of kodo millet. The optimum time for soaking, boiling, steaming, roasting, germination and fermentation was determined by analysing the changes in moisture content, equivalent diameter, bulk density, reducing sugar and total sugar content. The optimally processed kodo millet was analysed for its total polyphenol content, antioxidant activity and FTIR spectra for identification of unknown polyphenolic compounds. Result revealed that kodo millet could be soaked for 76-78 hours, boiled/steamed for 25-30 minutes, roasted for 15-20 minutes, fermented for 48 hours at 37°C and germinated for 72 hours at 37°C and 50% RH optimally. The various processing methods significantly affected the total polyphenol content. Among the processed samples, the highest polyphenol content and antioxidant activity was noted in germinated and boiled grain respectively. FTIR spectral analysis revealed the presence of polyphenolic compounds especially with mono substituted benzene ring in all processing conditions.

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### Introduction

Millet is in the family of cereals grown globally with differential importance across continents and within regions of the world. Millet is most often associated as the main ingredient in bird seed; it is not just “for the birds”. Creamy like mashed potatoes (or) fluffy like rice, millet is a delicious grain that can accompany many types of food. As with most grains, millet is available in markets throughout the year (Anderson et al, 2000). The nutritious cereals are represented by sorghum, pearl millet, finger millet, maize and minor millets like barnyard millet, proso millet, kodo millet and foxtail millet (Seetharama and Rao, 2004).

The kodo millet seeds are enclosed in coloured hulls, with colour depending on variety, and the seed heads themselves are held above the grassy plant on a spike like particle 14 to 16 inches long and are extremely attractive because of a remarkably hard, indigestible hull, this grain must be dehulled before it can be used for human consumption. Once out of the hull, millet grains look like tiny yellow spheres with a dot on one side where it was attached to the stem. The seeds are also rich in phytochemicals, including phytic acid, tannin, polyphenols (Railey, 2009).

Kodo millet in addition to nutritional benefits is rich in phytochemicals, including phytic acid which is believed to lower cholesterol and tannin, which is associated with cancer risk. Finger millet and kodo millet are well known for their antinutrient constituents such as trypsin inhibitors, phytates, phenols and tannins (www.chetday.com,2002). Higher antioxidant activity in the phenolic extract of kodo millet and finger millet than in other millets and cereals has been reported.

Small millets have potential benefits to mitigate (or) delay the onset of complications associated with diabetes. Kodo millet are rich source of phenolics, tannins and phytates, which can act as anti-oxidant (Prashant and Penumadu, 2005)

The study on effect of various processing conditions on total polyphenols content and antioxidant activity of kodo millet is still in its infancy. Hence the present investigation was planned with the objectives to optimize the processing methods of kodo millet, effect of optimized conditions on total polyphenol content, antioxidant activity, and FTIR spectra for identification unknown polyphenolic compounds.

#### Materials and methods

The selected kodo millet variety (CO3) was purchased from the local Gandhi market at Trichy district. The selected kodo millet was cleaned and winnowed manually for removal of dust and other foreign matters before it was being used for processing.

#### Determination of optimum processing conditions

The optimum time for soaking, boiling, steaming and roasting was determined by analyzing the changes in moisture content (moisture balance method), equivalent diameter and bulk density of the grain during 85 hours of soaking with continuous replacement of water at every 12 hours, 35 minutes of steaming in a steamer, 35 minutes of boiling and 25 minutes of roasting. The point at which the determined parameters reaches the peak and become stable would be considered as optimum time for processing.

The optimum time of fermentation and germination was determined by changes in reducing sugar and total sugar (Dinitro salicylic acid method - Sadasivam and Manickam, 2005)

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content during natural fermentation in a fermentor and germination of kodo millet in a germination container for 72 hours at 37°C and 25-27°C respectively. The point at which the determined parameters reach the maximum during fermentation and start raising the level in germination would be considered as optimum time of processing.

The optimum time of fermentation was also confirmed by the physical observation on appearance and disappearance of air bubbles over the surface of the broth as starting and ending point of fermentation. Then the kodo millet grain subjected to optimum time of each processing was dried, milled into flour and stored in LDPE package for further analysis.

#### **Determination of the total polyphenol content**

The total polyphenol content of raw and processed kodo millet flour prepared after processing by different methods, sieved through 40 mesh sieve (BSS) was analyzed using the method suggested by Ribeiro et al (2008).

#### **Determination of anti-oxidant activity**

Antioxidants are defined as molecules, present in small concentrations that prevent (or) reduce the extent of oxidative damage of biologically relevant molecules (Halliwell and Gutteridge, 1989; Ghorpade and Kadam, 1989). The free radical scavenging activity was evaluated by the DPPH method (Brand-Williams et al., 1995; Torres et al., 2006).

The DPPH test can provide information on the ability of a compound to donate an electron, the number of electrons, a given molecule can donate and on the mechanism of antioxidant action. In case where the structure of electron donor is not known (eg., a plant extract), this method can afford data on the reduction potential of unknown materials and this is also very convenient method for screening small antioxidant molecules because the reaction can be observed visually using common TLC and also its intensity can be analyzed by simple spectrophotometric assays.

The samples (0.1ml) were added to aliquots (3.9 ml) of solution made up with 4.8 mg DPPH in 200ml of MeOH and after vortexing the mixture was incubated for 1h at room temperature.

The initial concentration of DPPH, approximately 60µM, was calculated for every experiment from a calibration curve made by measuring the absorbance at 517 nm of standard samples of DPPH at different concentrations. The studied compounds were tested with MeOH as negative control and BHUT as positive control and absorbance at 517 nm was determined. The absorbance (A) of the control and samples was measured and the DPPH scavenging activity in percentage was determined as:

$$\text{DPPH scavenging activity (\%)} = \frac{[A_{\text{control}} - A_{\text{sample}}] / A_{\text{control}}}{1} \times 100$$

#### **Identification of polyphenolic compounds**

FT-IR measurements to identify polyphenolic compounds were made on a thermo electron corporation spectrophotometer (model-N ICOLET – 5700 FT-IR, USA).

Samples were applied on potassium bromide cell and spectra were run between 400 and 4000 cm<sup>-1</sup>. As per the obtained frequencies, relative intensity corresponding to the absorption of light was calculated. The functional groups were identified according to the absorbance band.

#### **Statistical analysis**

The collected data was systematically tabulated and analysed by statistical methods of descriptive frequencies, paired sample t-test and Pearson correlation coefficient using SPSS 11.0.

#### **Results and discussion**

#### **Optimum processing conditions of kodo millet**

The results revealed that the bulk density, moisture content and equivalent diameter were increased gradually with fluctuations and reached the peak and become stable at 76 hours of soaking; 25 minutes of boiling; 30 minutes of steaming and 20 minutes of roasting. The total sugar and reducing sugar content reaches the maximum during 48 hours fermentation and 72 hours of germination and then decreases. A similar change in total sugar content during fermentation was noted in cowpea studied by Akpapaunam and Achinewhu (2005). The chemical changes occurring during fermentation include an increase in free sugar indicating a partial breakdown of carbohydrates. An appreciable increase in starch content has been reported by Taur et al (1984) after fermentation in Sorghum. On the other hand, the total soluble sugars, total reducing sugars increased in fermentation (Kheterpaul and Chauhan, 1990; Soni and Sandhu, 1989).

#### **Effect of processing on total polyphenol content**

The result on total polyphenol content of processed and unprocessed kodo millet grain (Table 1) reveals that the total polyphenol content of unprocessed kodo millet grain was 5-6 times higher than the germinated grain and the total polyphenol content of processed kodo millet samples was significantly lower at p < 0.01 than the unprocessed sample. Similar observation i.e. reduction in total polyphenolic content was noted on processing of pearl millet cultivars by Eltayeb et al (2007). Among the processed samples, the highest polyphenol content was noted in germinated grain. From the results the descending order of the total polyphenol content in the kodo millet flour subjected to various processing treatments was germination > fermentation > boiling > steaming > roasting > soaking. The total polyphenol content during germination may be due to solubilization of tannins and the soluble tannin migrates from the seed coat to the core of the seeds as mentioned by Reema et al (2004).

#### **Effect of processing on antioxidant activity**

The result on antioxidant activity of processed and unprocessed kodo millet (Table 1) reveals that the antioxidant activity of BHT (0.1 mg/ml) which used as a standard for analysis was 37.47%. Among the processed samples, highest antioxidant activity (significant at p < 0.01) was noted in boiled grain which was also significantly higher than the antioxidant activity of unprocessed grain at p < 0.01. The steamed and fermented grain had exhibited significantly lowest antioxidant activity. The germinated kodo millet had similar antioxidant activity in comparison with BHT control. As per the Pearson correlation coefficient analysis, there was no significant correlation between total polyphenol content and antioxidant activity of kodo millet at p < 0.05. The obtained results were controversial to the result reported by the Dykes and Rooney (2006) and Hegde et al (2005) in barley and sorghum respectively.

#### **FTIR spectral analysis of kodo millet**

Infrared spectra of kodo millet flour subjected to various process conditions (figures 1-7) for identifying the polyphenolic compounds (Table 2) revealed that the vibrational band above 3600 cm<sup>-1</sup> may be due water molecule since water is a ubiquitous substance and found as common contaminant in many sub samples. As per the tentative assignment for functional group, a broad band was noticed at 3361.44 cm<sup>-1</sup> with very strong intensity for the unprocessed kodo millet flour which may be due to hydrogen bonded O-H group. This indicate the presence of diterpenes (phenolic compound) where further proven with the sharp peak at 2924.01 cm<sup>-1</sup> with very strong intensity which was assigned to CH<sub>3</sub> groups and symmetric CH<sub>3</sub>

stretching vibrations, ester carbonyl ( $1272\text{-}1050\text{ cm}^{-1}$ ) and phenyl ( $1600\text{ cm}^{-1}$ ) compounds.

The processing conditions cause a spectral shift in this band ( $3360\text{ cm}^{-1}$ ) except the germination process. The spectral shift in soaking process revealed  $\text{C} \equiv \text{CH}$  stretching indicate the  $\text{CH}_3$  compound; steaming and germination revealed very strong peak at  $3413.70\text{ cm}^{-1}$  indicate N-H stretching due to the presence amides; an aromatic system is probably present because low intensity of  $\text{CH}_2$  vibration. This is confirmed by the absorption at  $1600$  and  $850\text{-}700\text{ cm}^{-1}$ . The roasting revealed a very strong peak at  $3314.72\text{ cm}^{-1}$  indicate C-C stretching and boiling revealed very strong peak at  $3388.23\text{ cm}^{-1}$  indicate C-N stretch and the presence of aromatic amines. Though there was a spectral shift in this region of vibrations, no change in relative intensity of band and also revealed that the band at this frequency of  $3350\text{ cm}^{-1}$  may be aroused due to the polymeric structures.

The result inferred the presence of methyl group on aromatic ring with symmetric CH stretching by very strong peak at  $2924\text{ cm}^{-1}$  in all processing conditions with  $\pm 5\text{ cm}^{-1}$  variation. As per the FTIR-spectrum result on phenolic acids identified in the acidic extract of mature wheat straw, the free hydroxy benzoic acid compound accounted a peak at  $2924\text{ cm}^{-1}$  and also at  $1600\text{ cm}^{-1}$  (Smith, 1999). The present study also revealed the same results which confirm the presence of free hydroxyl benzoic acid compound in kodo millet flour.

The kodomillet flour samples subjected to soaking, germination, steaming, boiling and fermentation indicated the peak between  $2143\text{-}2150\text{ cm}^{-1}$  represent the presence of ketenes with  $\text{C} = \text{O}$  stretching at the region specially absent in untreated and roasted flour. The relative intensity of the compound was medium in soaking process, low in steaming and fermentation, and very low in germination and boiling process.

The peak at  $1600 \pm 5\text{ cm}^{-1}$  with very strong intensity in all processing conditions revealed the presence of hexa-substituted benzene with C-C stretching which may revealed the presence of polyphenolic compounds as P-hydroxy benzoic acid, gentisic acid, protocatechuic acid,  $\alpha$ ,  $\beta$ -dihydroferulic acid and trans-coumaric acid as indicated by Ram et al (2003).

The region below  $1500\text{ cm}^{-1}$  is rich in much absorption which was caused by bending vibrations and those resulting from the stretching vibrations of C-C, C-O and C-N bonds. The said region is called finger print region. The most diagnostic of these bands, the O-H stretching vibrations is typically found at  $3350 \pm 50\text{ cm}^{-1}$  and the in plane O-H bond occurs at  $1350 \pm 50\text{ cm}^{-1}$ . Any band due to an O-H bond will usually be broader than bond due to C-H bond. In coupling with O-H in plane bending if there is indication C-O tertiary between  $1150$  and  $1000\text{ cm}^{-1}$  may revealed the presence of alcohol. The phenol and tertiary alcohol's C-O stretching bond ranges do overlap a little, so it may be difficult to distinguish them.

Phenol is an example of a mono-substituted benzene ring (Smith, 1999). In the present study a very strong intensive peak between  $1355$  and  $1364\text{ cm}^{-1}$  indicate C-O stretching and O-H in plane bending vibrations which may reveal the presence of tertiary alcohol (or) phenol. But the presence of phenol as a mono substituted benzene ring was further confirmed by having peak at  $1151\text{ cm}^{-1}$  and at  $1154\text{ cm}^{-1}$  with very strong intensity; at  $1024\text{ cm}^{-1}$  and at  $1031\text{ cm}^{-1}$  with very strong intensity; between  $762$  and  $764\text{ cm}^{-1}$  with low intensity. In reference with identified phenolic acids in acidic extract of mature wheat strew by FTIR study, the absorption frequency between  $762$  and  $764\text{ cm}^{-1}$  revealed the presence of protocatechuic acid.

This result also supported by Clothup et al (1990) that in substituted benzene ring compound C-H in plane bending vibrations, are found in  $1600\text{-}1000\text{ cm}^{-1}$  which some times strongly mixed with C-C vibrations at  $1027\text{ cm}^{-1}$ . It was also noted that benzene compounds with  $\text{NO}_2$  substitutes revealed a prominent extra band near  $700\text{ cm}^{-1}$  which may thought to involve  $\text{NO}_2$  out plane bending interacting some what with out of plane aromatic CH wagging vibrations.

The absorption band between  $577\text{-}583\text{ cm}^{-1}$  indicated  $\text{NO}_2$  stretching,  $\text{NO}_2$  out plane wagging vibrations (Nitro group) and C-bromine stretching (Acyclic and aromatic bromo compounds).

On roasting, the peak at  $1246\text{ cm}^{-1}$ ,  $1151.5\text{ cm}^{-1}$ ,  $762.7\text{ cm}^{-1}$  and  $706.8\text{ cm}^{-1}$  was not observed. The peak at  $1151.5\text{ cm}^{-1}$  and  $706.8\text{ cm}^{-1}$  in spectrum of untreated kodomillet flour was not occurred in boiling process. The peak at  $706.8\text{ cm}^{-1}$  was also not occurred in steaming and fermentation process. This may indicate the deterioration of alcoholic (or) phenolic compounds. The peak indicating the presence of ketene group with  $\text{C} = \text{C} = \text{O}$  stretching ( $2150.5 \pm 5\text{ cm}^{-1}$ ) was unnoticed in unprocessed and roasted samples. The reduction in intensity of compound on processing was noticed at  $2150 \pm 5\text{ cm}^{-1}$ ,  $706.8 \pm 3\text{ cm}^{-1}$  and  $578 \pm 5\text{ cm}^{-1}$  may correspond to extraction of the compound into the solution on processing and the spectral shift at several peaks were consistent with depreciation of the phenolic group as observed by Ram et al 2003.

### Conclusion

Thus the optimum conditions of processing of kodo millet grain were 76-78 hours of soaking; 25-30 minutes of boiling; 25-30 minutes of steaming; 15-20 minutes of roasting; 48 hours of fermentation and 72 hours of germination to get maximum benefits. Though there was a reduction in total polyphenol content in all processing conditions, which was high in germination. While considering the antioxidant activity, boiling process indicated maximum activity. The total polyphenol content and antioxidant activity was not correlated significantly. FTIR spectral analysis revealed the presence of mono substituted benzene ring compound as phenol in all processing conditions.

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**Table – 1 Total polyphenol content and antioxidant activity of raw and Processed kodo millet**

S.No	Processing method	Polyphenol content	Antioxidant activity (%)
1	Unprocessed	3.2 ± 1.73	26.67±0.325
2	Germination	5.6 ± 1.10*	35.66±0.707 <sup>ns</sup>
3	Boiling	3.4 ± 0.20*	62.41±0.989 <sup>ns</sup>
4	Steaming	1.9 ± 0.10*	15.71±0.707 <sup>**</sup>
5	Roasting	1.4 ± 0.10*	23.67±0.282 <sup>*</sup>
6	Soaking	0.8 ± 1.17*	29.72±0.989 <sup>ns</sup>
7	Fermentation	4.2 ± 0.20*	15.71±0.707 <sup>**</sup>
8	BHT (0.1 mg/ml) – Positive control	-	37.47±0.707 <sup>**</sup>

\* Significant at  $p < 0.01$ , \*\* significant at  $p < 0.05$ , <sup>ns</sup>- not significant. The values in table are the average of two determinants

**Table 2: Phenolic compounds identified through FTIR spectra**

Identified phenolic compounds	UP	B	S	R	G	F	SO
Free hydroxy benzoic acid	p (s)	p (s)	p (s)	p (s)	p (s)	p (s)	p (s)
Ketenes with C=O stretching	-	p (vl)	p (l)	-	p (vl)	p (l)	p (m)
p- hydroxy benzoic acid, gentisic acid, $\alpha,\beta$ - dihydroferulic acid and trans-p-coumaric acid	p (s)	p (s)	p (s)	p (s)	p (s)	p (s)	p (s)
Tertiary alcohol/ phenolic or alcoholic compound	p (s)	-	-	-	p (s)	-	p (s)
Phenol as a monosubstituted benzene ring	p (s)	-	p (s)	-	p (s)	p (s)	p (s)
Prochatechuic acid	p (l)	p (l)	p (l)	-	p (l)	p (l)	p (l)
Nitro group and Acyclic aromatic bromo compounds	p (s)	p (m)	p (m)	p (s)	p (m)	p (m)	p (s)

p- presence of identified phenolic compounds, - absence of identified phenolic compounds, vl- very low intensity, l- low intensity, m- medium intensity, s- strong intensity, UP- Unprocessed, B-Boiled, S-Steamed, R-Roasted, G-Germinated, F-Fermented, SO- Soaked

FIG-1 FTIR-SPECTRA OF UNPROCESSED KODO FLOUR

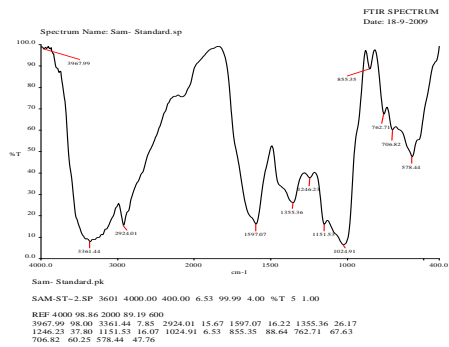


FIG-2 FTIR-SPECTRA OF FERMENTED KODOMILLET

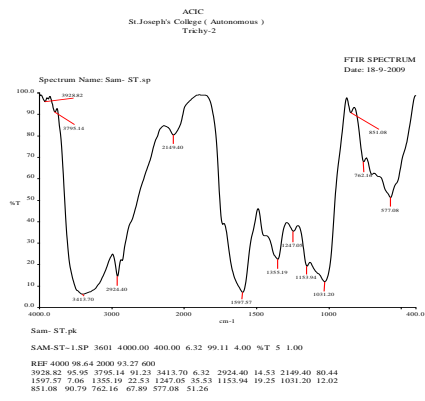


FIG-3 FTIR-SPECTRA OF SOAKED KODOMILLET FLOUR

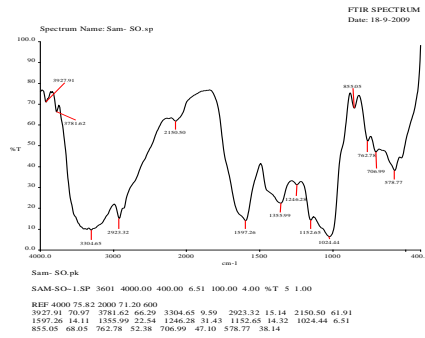


FIG-4 FTIR-SPECTRA OF GERMINATED KODOMILLET FLOUR

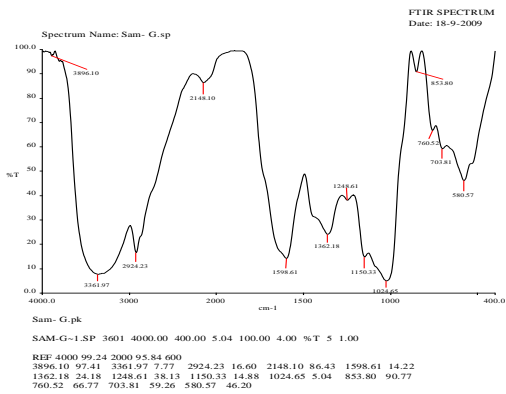


FIG-5 FTIR-SPECTRA OF STEAMED KODOMILLET FLOUR

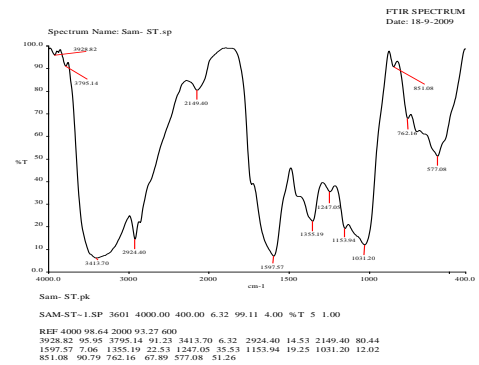


FIG-6 FTIR-SPECTRA OF BOILED KODOMILLET FLOUR

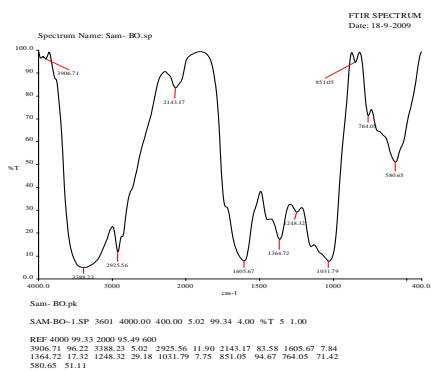


FIG-7 FTIR-SPECTRA OF ROASTED KODOMILLET FLOUR

